Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Original contribution

Comprehensive evaluation of bronchoalveolar lavage from patients with severe COVID-19 and correlation with clinical outcomes☆

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Summary Information on bronchoalveolar lavage (BAL) in patients with COVID-19 is limited, and clinical correlation has not been reported. This study investigated the key features of BAL fluids from COVID-19 patients and assessed their clinical significance. A total of 320 BAL samples from 83 COVID-19 patients and 70 non-COVID-19 patients (27 patients with other respiratory viral infections) were evaluated, including cell count/differential, morphology, flow cytometric immunophenotyping, and immunohistochemistry. The findings were correlated with clinical outcomes. Compared to non-COVID-19 patients, BAL from COVID-19 patients was characterized by significant lymphocytosis ($p < 0.001$), in contrast to peripheral blood lymphopenia commonly observed in COVID-19 patients and the presence of atypical lymphocytes with plasmacytoid/plasmablastic features ($p < 0.001$). Flow cytometry and immunohistochemistry demonstrated that BAL lymphocytes,

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including plasmacytoid and plasmablastic cells, were composed predominantly of T cells with a mixture of CD4+ and CD8+ cells. Both populations had increased expression of T-cell activation markers, suggesting important roles of helper and cytotoxic T-cells in the immune response to SARS-CoV-2 infection in the lung. More importantly, BAL lymphocytosis was significantly associated with longer hospital stay ($p < 0.05$) and longer requirement for mechanical ventilation ($p < 0.05$), whereas the median atypical (activated) lymphocyte count was associated with shorter hospital stay ($p < 0.05$), shorter time on mechanical ventilation ($p < 0.05$) and improved survival. Our results indicate that BAL cellular analysis and morphologic findings provide additional important information for diagnostic and prognostic work-up, and potential new therapeutic strategies for patients with severe COVID-19.

1. Introduction

Patients with coronavirus disease-2019 (COVID-19) have a wide spectrum of clinical presentations ranging from asymptomatic to fatal [1−8]. Pneumonia is the most common presentation in severe COVID-19, and acute respiratory distress syndrome (ARDS) is the major complication in critically ill patients [2,3,5,7]. The most common abnormal laboratory findings in hospitalized COVID-19 patients include peripheral blood lymphopenia and neutrophilia, elevated serum levels of D-dimer, amino transaminase, lactate dehydrogenase (LDH), and inflammatory markers, and abnormalities in coagulation tests [2,3,5−7,9−11]. Several laboratory features, such as lymphopenia and elevated D-dimer, have been associated with critical illness and mortality [2,5,6,8,9,11].

For patients with invasive mechanical ventilation, bronchoalveolar lavage (BAL) samples are often submitted for cell count and differential, in conjunction with molecular and microbiology testing, for evaluation of SARS-CoV-2 infectious status and diagnosis of secondary infections. To date, information on BAL cellular analysis and its clinical significance in patients with COVID-19 is limited. Two earlier single case reports have described an increase in lymphocyte percentage or exuberant plasmacytosis in BAL fluid in patients with severe COVID-19 [12,13]. A recent study of 20 patients with severe COVID-19 reported BAL lymphocytosis with plasmacytosis [14]. We first published the image of morphologically unusual atypical lymphocytes in BAL fluid in addition to their presence in the peripheral blood in patients with COVID-19 [15]. Similar findings were subsequently reported in a few patients by others [16,17]. Given the limited morphologic data and lack of clinical correlation, more comprehensive studies of BAL fluids in a larger cohort of patients are needed, and the results may provide important information for the diagnosis, prognosis, and potential new therapeutic strategies in patients with severe COVID-19.

The current study evaluated a total of 320 BAL samples submitted to our Hematology Laboratory for cell count and the differential between March 9 and May 7 of 2020, including 202 samples from 83 patients with confirmed COVID-19 and 118 samples from 70 non-COVID-19 patients who were initially suspicious for SARS-CoV-2 infection. The BAL cell count, differential, and morphologic features were compared between the two groups. Flow cytometric immunophenotyping and immunohistochemistry were also performed on a subset of patients with COVID-19 to further evaluate BAL lymphocyte populations. The findings were correlated with clinical outcomes, including length of hospital stay, duration on mechanical ventilation and in-hospital mortality.

2. Materials and method

2.1. Case selection

This study was approved by the Institutional Review Board (IRB) of Northwestern University. The BAL samples submitted to the Hematology Laboratory at Northwestern Memorial Hospital between March 9 and May 7 of 2020 for cell count and differential and indicated as patients with confirmed COVID-19 or patients under investigation for COVID-19 were collected for the study. Laboratory results were reviewed, including molecular testing for SARS-CoV-2, microbiology testing for the respiratory viral panel, BAL total cell count and differentials, and associated CBC and differentials. The clinical information, including diagnosis, length of hospital stay, duration on mechanical ventilation, and in-hospital mortality, were obtained from the electronic medical records.

2.2. Molecular testing for SARS-CoV-2

During the study period, two SARS-CoV-2 testing assays, the Center for Disease Control and Prevention (CDC) SARS CoV-2 rRT-PCR panel and Xpert Xpress CoV-2 (Cepheid Inc., Sunnyvale, CA, USA), were used at our institution. The two assays received Emergency Use Authorization (EUA) for the qualitative detection of the nucleic acid of SARS-CoV-2 in nasopharyngeal (NP) swab. Testing performance of the two assays for BAL was

validated in-house. Remel M4 viral transport system (Thermo Fisher Scientific Remel product, Lenexa, Ks, USA) was used for NP samples collection and transportation. Total RNA was isolated using the QIAamp MinElute Virus Spin Kit (Qiagen, Valencia, CA, USA). The CDC SARS CoV-2 rRT-PCR panel was performed using primers, probes, reagents, and procedures designated by CDC. The viral RNA was amplified by a one-step procedure using the TaqPath 1-step RT qPCR master mix and the CDC-designed primers and probes (2019-nCOV Kit, IDT Coralville, IA). The products were amplified on the Quant Studio 6 Flex system (Thermo Scientific, Waltham, MA, USA). The Xpert Xpress CoV-2 assay is a real-time RT-PCR-based assay for the detection of SARS-CoV-2 viral RNA. The test was performed with the Cepheid GeneXpert system following the manufacturer's instructions.

2.3. BAL total cell count and differential

The specimen processing, slide preparation, and cell count and differential were performed according to the standard laboratory procedure and followed the Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with COVID-19 published by the CDC. Briefly, the total cell count was determined using a hemocytometer. Two glass slides were prepared from each BAL sample for the differential count by centrifuging the cells onto the slides, and the slides were air-dried and stained with Wright-Giemsa stain.

2.4. Morphologic evaluation of BAL fluid

Morphologic evaluation of BAL fluids was performed by three pathologists (YC, JG, QC) without knowledge of clinical information and results of SARS-CoV-2 testing. The degree of atypical lymphocytes in the BAL samples was arbitrarily defined by the average number of atypical lymphocytes in a 20 × microscopic field by counting 10 fields: occasional (0.5−4 cells/20 ×), moderate (5−9 cells/20 ×), frequent (10−49/20 ×), and abundant (≥50 cells/20 ×).

2.5. Flow cytometric immunophenotyping

Flow cytometric analysis was performed on the BAL samples with Becton Dickinson Biosciences FACSCanto II flow cytometer (BD, Franklin Lakes, NJ, USA). Data analysis was performed using FACSDiva software (BD). The following antibodies were used in this study: CD45 Krome Orange (Beckman Coulter, clone J33), CD3 Alexa Fluor 700 (BD, clone UCHT1), CD56 FITC (BD, clone NCAM16.2), CD19 BV421 (BD, clone HIB19), CD4 Pe-Cy5.5 (Beckman Coulter, clone 13B8.2), CD8 Pe-Cy7 (Beckman Coulter, clone SFCI21Thy2D3), CD38 APC (BD, clone HB7), HLA-DR (BD, L243), and CD25 PE (BD, clone 2A3).

2.6. Immunohistochemistry

The air-dried cytospin slides were fixed in 95% alcohol for 30 min, and immunohistochemistry was performed on automated Ventana Benchmark (Ventana System, Tucson, AZ, USA) or Leica Bond Max (Leica Microsystems, Buffalo Grove, IL, USA) using the Bond polymer refine detection HRP (Leica Biosystems, DS9800) method. The following antibodies were used: prediluted anti-CD3 (clone: LN10; Leica), anti-CD20 (dilution 1:1000, clone: L26; DAKO), prediluted anti-CD19 (clone BT51E; Leica), prediluted anti-CD4 (clone: 4B12; Leica) and CD8 (dilution 1:40, clone: C8/144B; DAKO).

2.7. Statistical analysis

All statistical analyses were at the individual patient-specific level. Data from patients with multiple BAL tests during hospital stay were analyzed using two different approaches, the highest (maximum) and median values of each of the laboratory test results, in order to obtain valid statistical inferences using patient-level laboratory variables.

The statistical analyses of BAL cell counts and differentials, comparing the COVID-19, non-COVID-19, and non-COVID-19 with other respiratory viral infection groups, used a two-sample t-test unequal variances if no empirical evidence of non-Gaussian data distributions; otherwise, a nonparametric Wilcoxon two-sample rank test was used. Summary descriptive statistics were calculated for these three subgroups of patients for variables of interest. The 95% confidence intervals (CIs) for differences between pairs of groups, based on the t-test analysis, were derived, if appropriate. Statistical comparisons of the proportion of patients who were positive for BAL atypical lymphocytes (≥0.5 per 20 × microscopic field) in the COVID-19 versus non-COVID-19 patients and the proportion of in-hospital deaths among patients who were positive (≥0.5 per 20 × microscopic field) versus negative for atypical lymphocytes (<0.5 per 20 × microscopic field) were made using Pearson's Chi-squared test with Yates' correction, or Fisher's exact test if the numbers of patients were small.

Associations of BAL findings with clinical outcomes in COVID-19 patients were assessed using odds ratios for categorical outcome variables (in-hospital death versus discharged) and Spearman rank correlations for the laboratory data and continuous outcomes (length of hospital stay, length of mechanical ventilation). In addition, for the laboratory variables that were not highly correlated with each other, a generalized linear model was used to explore multivariable associations. For dichotomous clinical outcomes, a logistic regression model was used, and odds ratios and 95% CIs were obtained; for continuous clinical outcomes, linear regression was used, and corresponding p-values (based on a Wald test) were obtained.

3. Results

3.1. Patient characteristics

A total of 320 BAL samples from 153 patients submitted to the Hematology Laboratory at Northwestern Memorial Hospital for cell count and differential between March 9 and May 7 of 2020 were collected for the study. These included 202 samples from 83 patients with confirmed COVID-19 and 118 samples from 70 non-COVID-19 patients who were initially suspicious for COVID-19, including 27 patients with other respiratory viral infections.

The characteristics of 83 patients with COVID-19 are summarized in Table 1. These patients included 58 males and 25 females with a male to female ratio of 2.3, and age ranged from 21 to 90 years (median age 63 years). Clinical information was obtained in 80 of 83 patients. The underlying medical conditions listed by the CDC as risk or possible risk factors for severe illness from COVID-19 for an individual of any age group were identified in 65 of 80 (81.3%) patients and 7 of 80 (8.8%) patients, respectively. The risk factors included obesity ($30 \leq$ BMI <40 kg/m^2) in 21 (26.3%), severe (morbid) obesity (BMI ≥ 40 kg/m^2) in 16 (20.0%), type 2 diabetes in 22 (27.5%), serious cardiovascular diseases in 18 (22.5%), malignancies in 10 (12.5%; 7 with solid tumor, 2 with hematopoietic neoplasm, 1 with both), chronic obstructive pulmonary disease (COPD) in 6 (7.5%), chronic kidney disease in 6 (7.5%), and immunocompromised state due to solid organ transplant in 4 patients (5.0%; 2 with renal, 1 with lung and 1 with heart transplant). Of 83 patients with COVID-19, 3 had coinfections with other respiratory viruses, including influenza B, human rhinovirus, and human coronavirus, each in one patient.

The 70 non-COVID-19 patients included 41 males and 29 females with a male to female ratio of 1.4, and age ranged from 19 to 94 years (median age 60 years). The comorbidities were identified in 68 of 70 (97.1%) patients, including cardiovascular diseases (35.7%), malignancies (22.9%), chronic lung disease (21.4%), solid organ transplant (15.7%), type 2 diabetes (12.9%), chronic kidney disease (8.6%), chronic liver disease (2.9%), autoimmune or chronic inflammatory disease (2.9%) and morbid obesity (2.9%). In the non-COVID-19 group, 42 patients had various bacterial and/or fungal infections based on BAL cultures, including 16 patients with both bacterial and fungal infections, 22 patients with bacterial, and 4 patients with fungal infections. Twenty-seven patients had other respiratory viral infections, including 8 patients with influenza A or B, 1 with parainfluenza, 6 with human rhinovirus, 7 with human coronaviruses, 3 with human metapneumovirus, and 2 with respiratory syncytial virus infections. One patient had both influenza B and human rhinovirus infections. Sixteen of the 70 patients presented with acute hypoxic respiratory failure and abnormal imaging findings, but the respiratory viral panel and bacterial/fungal cultures were negative.

Table 1 Characteristics and comorbidities of patients with COVID-19.

Age	21−90 years (median: 63 years)
Sex	58 M; 25 F (M:F = 2.3)
Comorbidity with increased risk for severe COVID-19[a]	65/80 (81.3%)
Obesity ($30 \leq$ BMI <40)	21 (26.3%)
Morbid obesity (BMI ≥ 40)	16 (20.0%)
$<$50years	7/16 (43.8%)
$\geq$50 years	9/64 (14.1%)
Type 2 diabetes mellitus	22 (27.5%)
Serious cardiovascular disease	18 (22.5%)
Malignancies	10 (12.5%)
Chronic obstructive pulmonary disease (COPD)	6 (7.5%)
Chronic kidney disease	6 (7.5%)
Solid organ transplant	4 (5.0%)
Comorbidity with possible increased risk for severe COVID-19**	7/80 (8.8%)
Hypertension	6
Immunocompromised state	3
Liver disease	1
No significant comorbidity	7/80 (8.8%)
Coinfection with other respiratory virus	3/83 (3.6%)
Influenza B	1
Human Rhinovirus	1
Human coronavirus	1

[a] The underlying medical conditions with increased risk or possible increased risk for severe illness from COVID-19 for people of any age group are based on CDC publications (https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/people-with-medical-conditions.html).

Table 2 Comparison of lymphocyte percentage, absolute lymphocyte count, and atypical lymphocytes in bronchoalveolar lavage fluids between COVID-19 patients and non-COVID-19 patients.

BAL lymphocyte findings[a]	COVID-19 group[b] (n = 83)	Non-COVID-19 group[b] (n = 70)	Statistical analysis
Lymphocyte percentage[a]	n = 83	n = 69	
Highest count	25.0	3.0	$P < 0.001$
Median count	16.0	3.0	$P < 0.001$
Lymphocyte absolute count (k/μl)[a]	n = 83	n = 67	
Highest count	70.0	12.3	$p < 0.001$
Median count	39.4	8.7	$p < 0.001$
Patients with highest BAL lymphocyte count ≥ 15%	62/83 (74.7%)	9/69 (13.0%)	$p < 0.001$[c]
15−29%	28/62	5/9	
30−49%	21/62	4/9	
>50%	13/62	0	
Patients with atypical lymphocytes in BAL	60/83 (72.3%)	6/70 (8.6%)	$p < 0.001$[c]
Occasional (0.5−4 cells per 20)	26/60	6/6	
Moderate (5−9 cells per 20 ×)	11/60	0	
Frequent (10−49 cells per 20 ×)	17/60	0	
Abundant (≥50 cells per 20 ×)	6/60	0	

[a] The statistical analyses were at the individual patient-specific level with two approaches using individual patient's highest and median values of consecutive BAL samples submitted for cell count and differential during their hospital stay.

[b] The values of lymphocyte percentage and absolute count in the column represent median values derived from patients in the group.

[c] Pearson's Chi-squared test with Yates' correction and Fisher's exact test were used for statistical analysis. The remaining analyses were based on nonparametric Wilcoxon two-sample rank test.

3.2. Significant lymphocytosis in BAL in patients with COVID-19

Based on the published literature, BAL samples from healthy individuals contain mostly macrophages (80−90%), few lymphocytes (5−15%), neutrophils (≤3%), and eosinophils (<1%) [18,19]. An increase in lymphocyte differential to ≥15% is an uncommon occurrence and has been used for the evaluation of interstitial lung disease based on the Clinical Practice Guidelines of the American Thoracic Society [19].

As shown in Table 2 and Fig. 1, one of the most significant findings of the BAL cell count and differential in patients with COVID-19 was significant lymphocytosis. In the COVID-19 group, 62 of 83 (74.7%) patients had increased lymphocytes to ≥15% in at least one of the consecutive BAL samples collected during the hospital stay, whereas only 9 of 69 (13.0%) patients in the non-COVID-19 group had ≥15% lymphocytes ($p < 0.001$). Since many of our patients had multiple BAL samples collected for cell count and differential during the hospital stay, we chose to derive (calculate) two patient-level variables, highest and median values of cell percentage or absolute count, for statistical analysis. Comparison of the BAL lymphocyte percentage and absolute count between the two groups showed that patients with COVID-19 had significantly higher percentages ($p < 0.001$) and absolute count of lymphocytes ($p < 0.001$) than non-COVID-19 patients. The highest BAL lymphocyte percentages from 62 patients in the COVID-19 group included 28 patients between 15 and 29%, 21 between 30 and 49%, and 13 over 50%. As shown in Fig. 1, specific comparison to the subgroup of non-COVID-19 patients with other respiratory viral infection, patients with COVID-19 had significantly higher lymphocyte percentage and absolute count ($p < 0.001$). Only one of the 27 non-COVID-19 patients with influenza A pneumonia had mildly increased lymphocytes.

The remaining BAL cellular analyses demonstrated that the total cell counts, both the highest and median values, were substantially increased in both COVID-19 and non-COVID-19 patients, but there were no significant differences between the two groups. The neutrophil percentage and absolute count, both highest and median values, were also increased in both groups, but there were no significant differences between the two groups except that the non-COVID-19 patients had significantly higher median neutrophil percentage ($p < 0.001$), likely reflecting the overall higher neutrophil percentages over the disease course in non-COVID-19 patients. The highest monocyte percentage and absolute count were significantly higher in COVID-19 patients than non-COVID-19 patients ($p < 0.05$ for both variables), but there were no significant differences in the median values between the two groups.

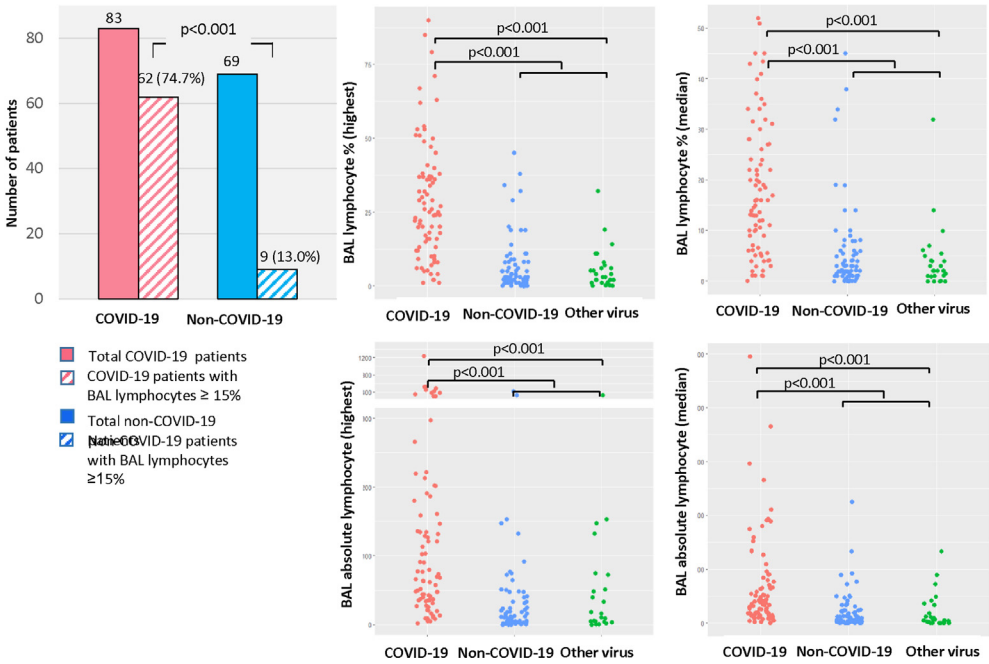

Fig. 1 Comparison of lymphocyte percentage and absolute count in bronchoalveolar lavage (BAL) fluids from COVID-19 and non-COVID-19 patients. Upper left: The proportion of patients with highest BAL lymphocyte percentage of ≥15% was significantly higher in the COVID-19 group than the non-COVID-19 group. Upper middle and right: Comparison of the two patient-specific variables (highest and median values) of BAL lymphocyte percentage (upper middle and right) showed that patients in the COVID-19 group had significantly higher BAL lymphocyte percentage than patients in the non-COVID-19 group, including the subgroup with other respiratory viral infections. Bottom panel: Comparison of two patient-specific variables (highest and median values) of BAL absolute lymphocyte count showed that patients in the COVID-19 group had significantly higher absolute lymphocyte count than patients in the non-COVID-19 group, including the subgroup with other respiratory viral infections.

3.3. Atypical lymphocytes with plasmacytoid or plasmablastic features in BAL fluids from patients with COVID-19

As shown in Table 2 and Fig. 2, the atypical lymphocytes with plasmacytoid or plasmablastic appearance were identified in BAL samples in 60 of 83 (72.3%) patients with COVID-19. These atypical lymphocytes ranged from medium to large with intensely basophilic cytoplasm and perinuclear clearance, resembling plasma cells or plasmacytoid lymphocytes, or displayed highly pleomorphic nuclei with prominent nucleoli resembling plasmablasts (Fig. 2). In contrast to COVID-19 patients, only 6 of 70 (8.6%) non-COVID-19 patients had rare to occasional atypical lymphocytes in BAL, and the lymphocytes demonstrated mild atypical features with no plasmablastic cells present. These 6 non-COVID-19 patients included 2 patients with influenza A pneumonia and 4 patients with nonviral-related illnesses.

The presence or absence and the number of atypical lymphocytes varied over the disease course but were most often identified in the initial BAL samples collected during the hospital stay. In order to objectively evaluate the degree of atypical lymphocytes, the average number of atypical lymphocytes in a 20 × microscopic field by counting 10 fields was used to arbitrarily define the degree of atypical lymphocytes as occasional (0.5−4 cells/20 ×), moderate (5−9 cells/20 ×), frequent (10−49 cells/20 ×) and abundant (≥50 cells/20 ×). As shown in Table 2, the highest number of atypical lymphocytes in BAL samples from each of the 60 COVID-19 patients included occasional in 26 (43.3%), moderate in 11 (18.3%), frequent in 17 (28.3%), and abundant in 6 (10.0%) patients. Of the 6 patients with abundant atypical lymphocytes, 3 had numerous atypical lymphocytes over 150 per 20 × field. In 6 of 70 non-COVID-19 patients, the counts of atypical lymphocytes were all near the lower limit defined as positive in this study (0.5 per 20 × field). None of these patients had atypical lymphocytes >2 per 20 × field, and none had highly atypical plasmablastic lymphocytes. Therefore, the presence of highly atypical lymphocytes, in combination with lymphocytosis in BAL, is a unique feature of COVID-19 pneumonia.

As described earlier in the results of BAL cell count and differential, neutrophilia and monocytosis were common in BAL fluids from patients with COVID-19, but there were no significant differences compared to the non-COVID-19 group with other respiratory viral or bacterial/fungal infections in our cohort of patients. Morphologically, no definite nuclear inclusions were identified in the

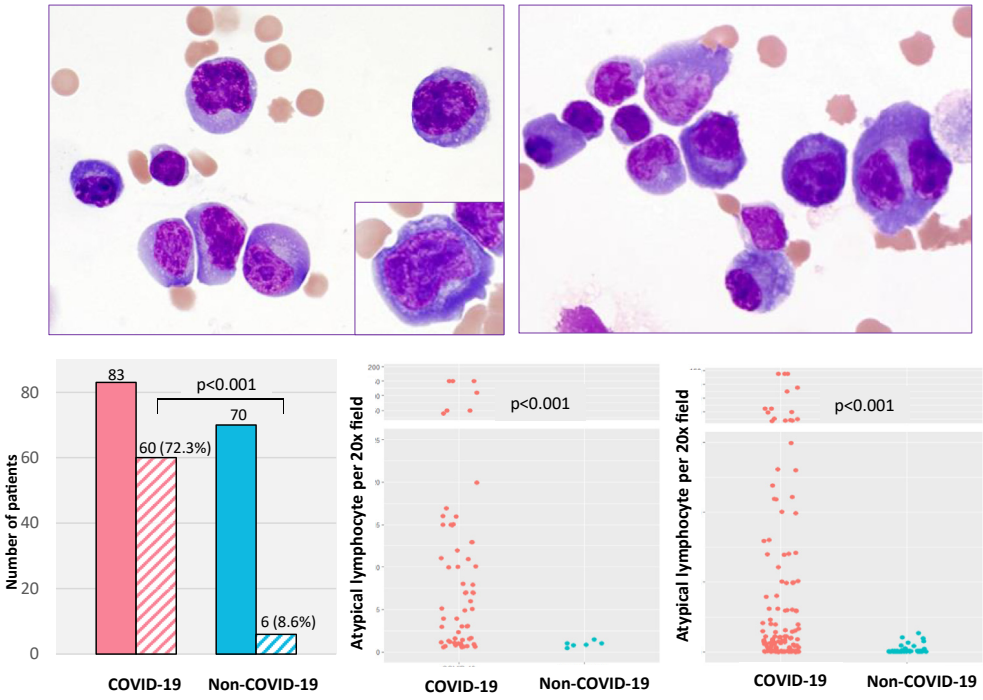

Fig. 2 Atypical lymphocytes with plasmacytoid or plasmablastic features in bronchoalveolar lavage (BAL) fluids from patients with COVID-19. Upper panel: Variable numbers of atypical lymphocytes were present in BAL fluids from patients with severe COVID-19. Images represent cases with abundant atypical lymphocytes with plasmacytoid or plasmablastic features (Magnification: 1000 ×). Lower left: The proportion of patients positive for atypical lymphocytes was significantly higher in the COVID-19 group than the non-COVID-19 group. The numbers of atypical lymphocytes in the 6 non-COVID-19 patients were all near the low limit defined as positive in this study, and none of the cases had highly atypical, plasmablast-like lymphocytes present Solid bar: total patients; Patterned bar: patients positive for atypical lymphocytes. Lower middle: Comparison of the highest count of atypical lymphocytes of BAL samples from the individual patient showed that patients with COVID-19 had significantly higher atypical lymphocyte count than non-COVID-19 patients. Lower right: Comparison of atypical lymphocyte count of all BAL samples from the individual patient showed that COVID-19 patients had significantly higher atypical lymphocyte count than non-COVID-19 patients.

monocytes/macrophages, type 2 pneumocytes, or respiratory epithelial cells in patients with COVID-19.

Here is an example of a patient that BAL morphologic findings provided additional helpful information for further clinical workup to clarify the patient's infectious status for SARS-CoV-2. The patient was an elderly female with a history of severe COPD and lung carcinoma who underwent a left lung transplant. The post-transplant course was complicated by antibody-mediated rejection, respiratory failure, and acute, chronic anemia. The respiratory viral panel on BAL was positive for non-SARS-CoV2 human coronavirus. Prior to esophagogastroduodenoscopy (EGD) to evaluate for gastrointestinal bleed, a BAL sample was sent for screening and came back unexpectedly positive for SARS-CoV-2. At that point, the patient isolation and contact tracing became very important, particularly given her long hospital stay and frequent interactions with the healthcare workers of various disciplines. The patient was

Table 3 Flow cytometric analysis of lymphocyte populations in bronchoalveolar lavage fluids from patients with COVID-19.

Lymphocyte subsets (n = 14)	Range (median)
T cells	69–94% (86.5%)
B cells	0–2% (0.9%)
NK cells	3–20% (9.9%).
CD4:CD8 ratio	0.3–2.5 (1.4).
Expression of activation markers in T cells (n = 9)	Positive/total cases (%); range of % positive T cell (median)
CD38+	9/9 (100%); 37–75% (64%)
HLADR+	8/9 (89%); 40–64% (52.8%)
CD25+	7/9 (78%); 30–60% (37.8%)

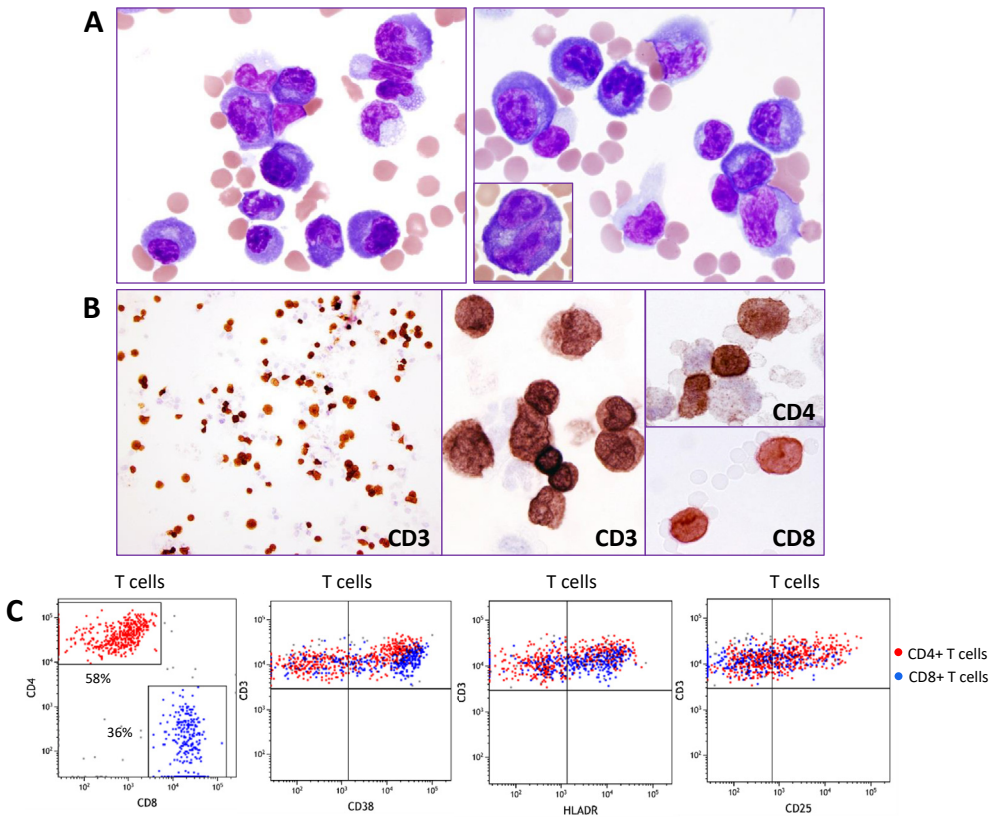

Fig. 3 Immunohistochemistry and flow cytometri-c analysis of bronchoalveolar lavage (BAL) fluids from a representative patient with severe COVID-19. A. This patient had numerous atypical lymphocytes (>150 per 20 × field) with plasmacytoid or plasmablastic features (Magnification: 1000 ×). B. Left: Low-power view of immunohistochemistry demonstrating the vast majority of lymphocytes were CD3+ T cells. Middle: The atypical lymphocytes, including those with plasmacytoid or plasmablastic features, were positive for CD3. Right: The atypical lymphocytes consisted of a mixture of CD4+ or CD8+ T cells. C. Flow cytometric analysis demonstrated that the T cells had a normal CD4 (red) to CD8 (blue) ratio of 1.6. Significant proportions of CD4+ or CD8+ T-cells were positive for T-cell activation markers, CD38, HLA-DR, and CD25.

transferred to COVID-19 ICU. Multiple repeat tests on both BAL and nasopharyngeal swabs were negative. A retrospective review of 5 BAL samples from the patient was performed and showed no atypical lymphocytes or increased lymphocyte percentage in any of the samples. Given the patient's clinical features and laboratory findings, the initial SARS-CoV-2 test was considered false positive. A subsequent COVID-19 IgG antibody test was also negative.

3.4. Flow cytometric and immunohistochemical analysis of lymphocytes in BAL fluids from patients with severe COVID-19

Flow cytometric analysis was performed on 14 BAL samples from 13 patients with COVID-19 to characterize the lymphocyte populations. As shown in Table 3, the lymphocytes of all 14 samples were composed predominantly of CD3+ T cells (69−94%; median 86.5%) and NK cells (3−20%; median 9.9%). CD19+ B cells were rare to absent (0−2%; median 0.9%). The CD4 to CD8 ratio ranged from 0.3 to 2.5 (median: 1.4). The lymphocyte activation markers, CD38, HLA-DR, and CD25, were assessed on nine samples from 9 patients. A significant proportion of T cells (40−80%) expressed bright CD38 (9/9, 100%), HLA-DR (8/9, 88.9%), and CD25 (7/9, 77.8%). Seven of the 9 patients were positive for all three markers, and the remaining 2 patients were positive for two of the three markers. Flow cytometric analysis also emonstrated that both CD4+ and CD8+ T-cell subsets expressed activation markers (Fig. 3).

Immunohistochemical stains were performed on the cytospin slides of 12 BAL samples from 12 COVID-19 patients. The lymphocytes consisted predominantly of CD3+ T cells (>95%) with absence or very rare CD19+ or CD20+ B cells (≤2%). The CD4 to CD8 ratio ranged from 0.3 to 2.0 (median: 1.0). The large atypical lymphocytes with plasmacytoid or plasmablastic features were virtually all CD3+ with no CD19+ or CD20+ large atypical cells seen. Fig. 3 illustrates the cellular morphology, immunohistochemistry, and flow cytometry results of BAL samples from a representative patient with severe COVID-19.

Table 4 Associations of lymphocyte percentage, absolute lymphocyte count, and atypical lymphocyte with clinical outcomes in patients with COVID-19.

Clinical outcomes	Lymphocyte findings	Statistical analysis	
		Highest value	Median value
Length of hospital stay (days)[a]	Lymphocyte percentage	***p = 0.006 (r = 0.323);***	*p = 0.947 (r = -0.008)*
	Lymphocyte absolute count	***p < 0.001 (r = 0.419);***	*p = 0.423 (r = 0.010)*
	Atypical lymphocyte count	*p = 0.587 (r = 0.067);*	***p = 0.013 (r = -0.296)***
Length on mechanical ventilation (days)[a]	Lymphocyte percentage	***p = 0.028 (r = 0.263);***	*p = 0.700 (r = -0.047)*
	Lymphocyte absolute count	***P = 0.016 (r = 0.292);***	*p = 0.867 (r = 0.021)*
	Atypical lymphocyte count	*P = 0.348 (r = 0.115);*	***p = 0.039 (r = -0.249)***
In-hospital mortality[b]	Lymphocyte percentage	*p = 0.192;*	*p = 0.535*
	Lymphocyte absolute count	*p = 0.933;*	*p = 0.444*
	Atypical lymphocyte count	*p = 0.178;*	*p = 0.338*
	Atypical lymphocyte negative vs positive[c]	*p = 0.094* (Mortality: 26.1% vs 8.8%)	

[a] Spearman rank correlation test.
[b] Odds ratios for categorical outcome variables, and.
[c] Pearson's Chi-squared test with Yates' correction were used for statistical analysis.

3.5. Correlation of BAL findings with clinical outcomes in patients with severe COVID-19

All 83 patients with COVID-19 in this study were severely ill and required intubation and mechanical ventilation. At the end of the study period, 71 patients (85.5%) recovered and were discharged from the hospital, 1 patient was still in the hospital, and 12 patients (14.5%) died during the hospital stay. The length of hospital stay ranged from 9 to 82 days (median: 29 days), and the duration of mechanical ventilation ranged from 3 to 76 days (median: 14 days).

The BAL findings in patients with COVID-19 were correlated with clinical outcomes. As shown in Table 4, the levels of highest BAL lymphocyte percentage and absolute count were significantly associated with longer hospital stay $p = 0.006$ and $p < 0.001$, respectively) and longer requirement for mechanical ventilation (both $p < 0.05$). Interestingly, the median atypical (activated) lymphocyte count was inversely correlated with length of hospital stay and length on mechanical ventilation (both $p < 0.05$). Additionally, patients without atypical (activated) lymphocytes in BAL fluids had higher in-hospital mortality (26.1%) than those with atypical lymphocytes (8.8%), although it did not reach statistical significance ($p = 0.09$). The remaining values in the BAL cellular analysis, including total cell count, neutrophil percentage and absolute count, and monocyte percentage and absolute count, showed no significant association with the clinical outcomes measured by the three parameters in this study.

4. Discussion

The common abnormal laboratory findings of hospitalized patients with COVID-19 include peripheral blood lymphopenia and neutrophilia, elevated D-dimer, amino transaminase, LDH, inflammatory markers, and abnormal coagulation tests. Some of the laboratory findings are associated with poor clinical outcomes. We previously reported results from a hierarchical clustering analysis of hematologic and biochemical results of 973 patients with COVID-19 and subclassified the patients into five risk clusters [20]. It is well recognized that peripheral blood lymphopenia is the most common hematologic finding in patients with COVID-19 [2,5–8,10,11], and more profound lymphopenia is associated with severe illness and disease progression [5,11,21]. We and others reported the presence of atypical lymphocytes with plasmacytoid features in the peripheral blood in patients with COVID-19 [15,22,23]. The presence of these atypical lymphocytes together with lymphopenia may provide important clues to further diagnostic workup for SARS-CoV-2 infection [15].

Although there have been overwhelming numbers of publications on COVID-19 since the SARS-CoV-2 pandemic in the spring of 2020, information on BAL findings and their clinical significance is limited. Some earlier case reports addressed the importance of molecular testing on BAL samples to confirm COVID-19 pneumonia in clinically highly suspected patients with consecutive negative nasopharyngeal swab results [24–26]. Others described increased lymphocyte percentage or exuberant plasmacytosis in BAL fluid in two patients with severe COVID-19 [12,13]. In addition to lymphopenia and atypical lymphocytes in the peripheral blood, morphologic findings of atypical lymphocytes in BAL fluid were also reported in a few patients by us and others [15–17]. A recent study reported lymphocytosis and plasmacytosis in patients with severe COVID-19 [14]. So far, information on BAL findings in COVID-19 patients is largely based on case reports and relatively small series, and the clinical association has not been investigated.

Our current study of 202 BAL samples from 83 patients with severe COVID-19 and 118 samples from 70 non-COVID-19 patients showed that BAL fluid from severe COVID-19 patients was characterized by lymphocytosis with significantly higher lymphocyte percentages and absolute lymphocyte counts than patients in the non-COVID-19 group, including those with other respiratory viral infections. Another striking feature was the presence of atypical lymphocytes with a plasmacytoid or plasmablastic appearance in the majority of patients with severe COVID-19. Additionally, neutrophilia and monocytosis were also common in BAL fluids from patients with COVID-19, but the findings were not significantly different from the non-COVID-19 patients with other respiratory viral or bacterial/fungal infections in our study. Nuclear inclusions in alveolar macrophages have been described in a patient with severe COVID-19,[13] but we did not identify convincing inclusions in cells of BAL fluids from patients with COVID-19. Our comparative studies demonstrate that BAL lymphocytosis in combination with highly atypical lymphocytes is a characteristic feature of severe COVID-19 pneumonia. Thus, for patients on prolonged mechanical ventilation for non-COVID-19-related illnesses and prior negative SARS-CoV-2 testing, the findings of new BAL lymphocytosis and the presence of highly atypical lymphocytes with plasmablastic features should raise clinical suspicion and prompt repeat molecular testing for SARS-CoV-2. As demonstrated in the case example described in the result section, lack of lymphocytosis and atypical lymphocytes in multiple consecutive BAL samples led us to raise the suspicion for a false positive molecular testing result in a post-lung transplant patient with a new, clinically unexpected positive molecular testing for SARS-CoV-2. This suspicion was confirmed by multiple repeat negative molecular tests and subsequent negative antibody testing.

In the hematology laboratory, atypical lymphocytes, often associated with absolute lymphocytosis, are most commonly seen in the peripheral blood in patients with acute EBV infections. The atypical lymphocytes in BAL fluids from patients with severe COVID-19 had some morphologic differences from those seen in blood in EBV infection. They often exhibit intensely basophilic cytoplasm with perinuclear clearance, resembling plasma cells or plasmacytoid lymphocytes, or displayed highly pleomorphic nuclei with prominent nucleoli resembling plasmablasts. Flow cytometric analysis demonstrated that the vast majority of lymphocytes in BAL fluid were T cells, including both CD4+ and CD8+ T-cells; B cells were virtually absent. Further analysis demonstrated that both CD4 and CD8 subsets expressed high levels of T-cell activation markers. Immunohistochemistry on the cytospin slides confirmed that the atypical lymphocytes with plasmacytoid or plasmablastic features were CD4+ or CD8+ T cells. Our findings suggest that the previously reported exuberant plasmacytosis in BAL fluids from patients with severe COVID-19 likely represents activated T cells rather than plasmacytoid B cells or plasma cells.

Based on the literature, the median CD4 to CD8 ratio in BAL fluid in healthy adults is about 1.5. Our patients with COVID-19 had significantly increased lymphocytes in BAL fluids compared to non-COVID-19 patients, but the CD4 to CD8 ratio did not differ significantly from that in healthy individuals. Multiple studies have demonstrated that a decline in peripheral blood T cells or CD4+ and CD8+ subsets are associated with a higher risk of COVID-19 infection, severe disease, or in-hospital death [11,21,27–29]. In a study of 25 patients with COVID-19, Ganji et al. reported no significant difference in CD4 to CD8 ratio and CD4 mean fluorescence intensity (MFI) in peripheral blood T cells compared to normal individuals, but the CD8 MFI was significantly increased, suggesting that hyperactivation of cytotoxic T cells played an important role in the immune response to SARS-CoV-2 infection [30]. A study by Giamarellos-Bourboulis et al. demonstrated a significant decrease in HLA-DR expression on CD4+ T cells in patients with severe COVID-19 and plasma from COVID-19 patients inhibited HLA-DR expression, indicating that the T-cell activation and effective function were compromised in patients with severe COVID-19. In our current study, the T cells in BAL fluids from patients with severe COVID-19 demonstrated apparent morphologic and immunophenotypic evidence of activation as manifested by morphologically atypical (activated) T cells and expression of activation markers (CD38, HLA-DR, CD25) in both CD4 and CD8 T-cell populations. The results provided evidence that both helper and cytotoxic T cells play important roles in the immune response to SARS-CoV-2 infection in the lung. Thevarajan et al. reported the kinetics of immune response in relation to clinical and virological features in a patient with COVID-19 and found that a rapid increase in the coexpression of HLA-DR and CD38 on CD8+ T cells was detected before symptomatic recovery [31]. Thus, flow cytometric analysis on peripheral blood and/or BAL fluid for T-cell activation markers may provide important information for predicting a patient's outcome. Our study showed that flow cytometric analysis for T-cell subsets and T-cell activation markers as part of clinical BAL evaluation for patients with severe COVID-19 is feasible, but immunophenotyping of consecutive BAL samples to evaluate T-cell properties in relation to clinical features over disease course was not performed in this study.

Our study first demonstrated the significant correlations of BAL findings with clinical outcomes in patients with severe COVID-19. Two patient-level variables (highest and median values) derived from all samples from individual patients were used for statistical analysis. The results showed that both the highest lymphocyte percentage and absolute lymphocyte count were significantly associated with longer hospital stay and longer requirement for

mechanical ventilation, whereas the median atypical lymphocyte count was inversely associated with length of hospital stay and length on mechanical ventilation. Additionally, the in-hospital mortality was higher in patients who lacked atypical lymphocytes than patients with atypical lymphocytes in BAL fluids, although it did not reach statistical significance. The presence of atypical (activated) lymphocytes likely reflects the active T-cell response to SARS-CoV-2 infection in the lung and may explain the associated better outcomes relative to patients without atypical lymphocytes. It is noted that a very recent publication on peripheral blood samples demonstrated that the presence of atypical lymphocytes in blood showed correlation with a better prognosis in patients with COVID-19 [32], which is in concordance with our findings in the BAL fluids in patients with severe COVID-19.

Lastly, the demographics and risk factors of our cohort of patients with severe COVID-19 are of clinical interest. The overall demographics and underlying medical conditions of the COVID-19 patients in our cohort are comparable with the published data. A notable difference is the high percentage of patients with morbid obesity (20%) in our cohort, which is significantly higher than that (8%) reported by Klang et al. in a large study, which showed that the mortality for COVID-19 was significantly associated with severe obesity in patients younger than 50 years [33]. A significant inverse correlation between age and BMI was also found in hospitalized patients with COVID-19 in another study [34]. Similarly, in our cohort of patients, the proportion of patients with severe obesity was significantly higher in younger patients (<50 years; 43.8%) than in older patients (≥50 years; 14%). The finding supports that severe obesity is one of the major risk factors for severe COVID-19 in younger patients.

In summary, our comparative study demonstrated that BAL fluid from patients with severe COVID-19 was characterized by lymphocytosis and atypical lymphocytes with plasmacytoid/plasmablastic morphology. Immunophenotyping provided evidence that both helper and cytotoxic T cells play important roles in the immune response to SARS-CoV-2 infection in the lungs. More importantly, we have shown that some of the BAL findings were significantly associated with clinical outcomes. Our results indicate that BAL cellular analysis and morphologic findings provide important information for diagnostic and prognostic workup and potential new therapeutic strategies for patients with severe COVID-19.

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